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5 NOVEL ~~PHENOL~~ OXIDIZING ~~ENZYME~~ ENZYMES

Field of the Invention

The present invention relates to novel phenol oxidizing enzymes, in particular, novel phenol oxidizing enzymes derived from strains of *Stachybotrys* and novel strains of the genus *Stachybotrys* producing these enzymes. The present invention provides methods and host cells for expressing *Stachybotrys* phenol oxidizing enzymes as well as methods for producing expression systems. The present invention also relates to methods for modifying a colored compound and dye transfer prevention during fabric washing. Moreover the invention relates to an enzymatic detergent composition for stain bleaching or anti dye transfer.

Background of the Invention

Phenol oxidizing enzymes function by catalyzing redox reactions, i.e., the transfer of electrons from an electron donor (usually a phenolic compound) to molecular oxygen (which acts as an electron acceptor) which is reduced to H_20. While being capable of using a wide variety of different phenolic compounds as electron donors, phenol oxidizing enzymes are very specific for molecular oxygen as the electron acceptor.

Phenol oxidizing enzymes can be utilized for a wide variety of applications, including the detergent industry, the paper and pulp industry, the textile industry and the food industry. In the detergent industry, phenol oxidizing enzymes have been used for preventing the transfer of dyes in solution from one textile to another during detergent washing, an application commonly referred to as dye transfer inhibition.

Most phenol oxidizing enzymes exhibit pH optima in the acidic pH range while being inactive in neutral or alkaline pHs.

Phenol oxidizing enzymes are known to be produced by a wide variety of fungi, including species of the genii Aspergillus, Neurospora, Podospora, Botytis, Pleurotus, Fomes, Phlebia, Trametes, Polyporus, Rhizoctonia and Lentinus. However, there remains a need to identify and isolate phenol oxidizing enzymes, and organisms capable of naturally-producing phenol oxidizing enzymes, which present pH optima in the alkaline range for use in detergent washing methods and compositions.

Summary of the Invention

The present invention relates to novel phenol oxidizing enzymes obtainable from *Stachybotrys* which are capable of modifying the color associated with dyes and colored compounds having different chemical structures, in particular at neutral or alkaline pH. Based on their color modifying ability, phenol oxidizing enzymes of the present invention can be used, for example, for pulp and paper bleaching, for bleaching the color of stains on fabric and for anti-dye transfer in detergent and textile applications. In one aspect of the present invention, the phenol oxidizing enzyme is able to modify the color in the absence of an enhancer. In another aspect of the present invention, the phenol oxidizing enzyme is able to modify the color in the presence of an enhancer.

In one embodiment of the present invention, the phenol oxidizing enzymes are obtainable from *Stachybotrys*. In another embodiment, the *Stachybotrys* enzymes are selected from strains of the group consisting of *S.* parvispora, including, in particular, *S.* parvispora var. hughes MUCL 38996; strains of the species *S.* chartarum including, in particular, *S.* chartarum MUCL 38898; S. parvispora MUCL 9485; S. chartarum MUCL 30782; S. kampalensis MUCL 39090; S. theobromae MUCL 39293; and strains of the species S. bisbyi, S. cylindrospora, S. dichroa, S. oenanthes and S. nilagerica. In one aspect, the present invention provides a phenol oxidizing enzyme which has molecular weight of about 38 kD as measured by SDS polyacrylamide gel electrophoresis (PAGE). In another aspect, the present invention provides a phenol oxidizing enzyme which has a molecular weight of about 30.9 kD as measured by SDS polyacrylamide gel electrophoresis.

When partially purified phenol oxidizing enzyme obtained from a strain of *S.* parvispora or *S.*chartarum was boiled and subjected to SDS polyacrylamide gel electrophoresis, three molecular weight species were observed. For phenol oxidizing enzyme obtained from *S.* parvispora MUCL 38996, the three molecular weight species were about 70 kD, 45 kD and 22.1 kD. For phenol oxidizing enzyme obtained from *S.* chartarum MUCL 38898, the three molecular weight species were about 58.4 kD, 46.1 kD and 19.7 kD. The present invention encompasses any phenol oxidizing enzyme activity inherent to any of these molecular weight species alone or in combination with at least one other of the molecular weight species. The present invention also encompasses any phenol oxidizing enzyme which exhibits an

increase in apparent molecular weight after boiling, wherein the molecular weight is determined by SDS-polyacrylamide gel electrophoresis.

The present invention also encompasses phenol oxidizing enzymes which are capable of modifying the color associated with dyes or colored compounds and which have at least one antigenic group in common with phenol oxidizing enzyme naturally-produced by S. parvispora MUCL 38996 and/or the phenol oxidizing enzyme naturally-produced by S. chartarum MUCL 38898 as measured by the Ouchterlony technique in which a positive enzyme exhibits an immunoprecipitation line. In one embodiment, the immunoprecipitation line is Type 1 line. In one embodiment, the phenol oxidizing enzyme having at least one antigenic group in common with phenol oxidizing enzyme naturally produced by S. parvispora MUCL 38996 is obtainable from *Stachybotrys*. The present invention also encompasses *Stachybotrys* phenol oxidizing enzyme mutants as long as the mutant is able to modify the color associated with dyes or colored compounds.

In yet another embodiment, the present invention provides an isolated polynucleotide encoding a phenol oxidizing enzyme obtainable from Stachybotrys wherein said polynucleotide comprises a nucleic acid sequence having at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and at least 95% identity to SEQ ID NO:1 or SEQ ID NO:3 as long as the polynucleotide encodes a phenol oxidizing enzyme capable of modifying the color associated with dyes or colored compounds. The present invention also encompasses polynucleotide sequences that are capable of hybridizing under conditions of intermediate to high stringency to the polynucleotide shown in SEQ ID NO:1 or SEQ ID NO:3 or which are complementary thereto. The present invention also provides polynucleotides that encode the amino acid sequence as shown in SEQ ID NO:2. In a preferred embodiment, the polynucleotide has the nucleic acid sequence as shown in SEQ ID NO:1 or SEQ ID NO:3. The present invention also provides expression vectors and host cells comprising polynucleotides of the present invention.

The present invention additionally relates to methods for producing a phenol oxidizing enzyme obtainable from *Stachybotrys*. Accordingly, the present invention provides a method for producing said enzyme comprising the steps of obtaining a host cell comprising a polynucleotide encoding said phenol oxidizing enzyme

obtainable from *Stachybotrys* wherein said enzyme has at least 65% identity to the amino acid sequence disclosed in SEQ ID NO:2; culturing said host cell under conditions suitable for the production of said phenol oxidizing enzyme; and optionally recovering said phenol oxidizing enzyme produced. The present invention also provides a method for producing a phenol oxidizing enzyme comprising the step of culturing a recombinant host cell characterized by the expression of a polynucleotide encoding a phenol oxidizing enzyme obtainable from Stachybotrys wherein said enzyme has at least 65% identity to the amino acid having the sequence as shown in SEQ ID NO:2 and optionally recovering said phenol oxidizing enzyme. In one embodiment, the polynucleotide is present on a replicating plasmid and in another embodiment is integrated into the host genome.

In one embodiment, the polynucleotide comprises the sequence as shown in SEQ ID NO:1. In another embodiment, the polynucleotide comprises the sequence as shown in SEQ ID NO: 3. In a further embodiment, the polynucleotide is capable of hybridizing to SEQ ID NO:1 or SEQ ID NO:3 under conditions of intermediate to high stringency or is complementary to SEQ ID NO:1 or SEQ ID NO:3.

The present invention also provides a method for producing a recombinant host cell comprising a polynucleotide encoding a phenol oxidizing enzyme of the present invention comprising the step of introducing a polynucleotide encoding said phenol oxidizing enzyme obtainable from Stachybotrys and having at least 65% identity to the amino acid sequence disclosed in SEQ ID NO:2 into a host cell; and optionally culturing said host cell under conditions suitable for the production of said phenol oxidizing enzyme. In one embodiment, the polynucleotide comprises the sequence as shown in SEQ ID NO: 1. In another embodiment, the polynucleotide comprises the sequence as shown in SEQ ID NO:3. In a further embodiment, the polynucleotide is capable of hybridizing to SEQ ID NO:1 or SEQ ID NO:3 under conditions of intermediate to high stringency or is complementary to SEQ ID NO:1 or SEQ ID NO:3.

In one aspect of the present invention, the recombinant host cell comprising a polynucleotide encoding a phenol oxidizing enzyme includes filamentous fungus, yeast and bacteria. In one embodiment, the host cell is a filamentous fungus including Aspergillus species, Trichoderma species and Mucor species. In a

preferred embodiment, the filamentous fungus host cell includes A. niger var. awamori and T. reseei.

In another embodiment of the present invention, the host cell is a yeast which includes Saccharomyces, Pichia, Hansenula, Schizosaccharomyces, Kluyveromyces and Yarrowia species. In yet a another embodiment, the Saccharomyces species is S. cerevisiae. In an additional embodiment, the host cell is a gram positive bacteria, such as a Bacillus species, or a gram negative bacteria, such as an Escherichia species. The present invention also encompasses methods for purifying the phenol oxidizing enzyme from such host cells.

Also provided herein are detergent compositions comprising the amino acid having a sequence at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the phenol oxidizing enzyme having the amino acid sequence disclosed in SEQ ID NO:2 as long as the enzyme is capable of modifying the color associated with dyes or colored compounds. In one preferred embodiment, the amino acid has the sequence as shown in SEQ ID NO: 2. In another preferred embodiment, the phenol oxidizing enzyme is encoded by a polynucleotide comprising the sequence as shown in SEQ ID NO: 1. In another embodiment, the phenol oxidizing enzyme is encoded by a polynucleotide comprising the sequence as shown in SEQ ID NO:3. In a further embodiment, the polynucleotide is capable of hybridizing to SEQ ID NO:1 or SEQ ID NO:3 under conditions of intermediate to high stringency or is complementary to SEQ ID NO:1 or SEQ ID NO:3.

The present invention also encompasses methods for modifying the color associated with dyes or colored compounds which occur in stains on fabric, comprising the steps of contacting the fabric with a composition comprising an amino acid sequence having at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identity to the phenol oxidizing enzyme having the amino acid sequence disclosed in SEQ ID NO:2 as long as the enzyme is capable of modifying the color associated with dyes or colored compounds. In one embodiment of the method, the amino acid has the sequence as shown in SEQ ID NO:2. In one aspect of the method, the pH optimum is between 5.0 and 11.0, in another aspect, the pH optimum is between 7 and 10.5 and in yet another aspect the pH optimum is between 8.0 and 10. In a further aspect of the method, the optimum temperature is between 20 and 60 degrees C. and in another aspect between 20

and 40 degrees C. The present invention also provides methods for preventing dye transfer in detergent and textile applications.

Also provided herein are detergent compositions comprising a *Stachybotrys* phenol oxidizing enzyme of the present invention alone or in combination with an enhancer and other detergent ingredients, including proteases, amylases and/or cellulases.

Enhancers which can be used in detergent compositions of the present invention include but are not limited to phenothiazine-10-propionic acid (PPT), 10-methylphenothiazine (MPT), phenoxazine-10-propionic acid (PPO), 10-methylphenoxazine (MPO), 10-ethylphenothiazine-4-carboxylic acid (EPC) acetosyringone, syringaldehyde, methylsyringate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate (ABTS) and 4-Hydroxy-4-biphenyl-carboxylic acid or derivatives thereof.

Brief Description of the Drawings

Figure 1 illustrates the pH profile of the oxidation of various chromophores by *Stachybotrys* parvispora phenol oxidizing enzyme.

Figure 2 illustrates the pH profile of Direct Blue1 bleaching as a comparison between *Stachybotrys* parvispora phenol oxidizing enzyme and Myrothecium verrucaria bilirubin oxidase.

Figure 3 illustrates the molecular weight of *Stachybotrys* chartarum phenol oxidizing enzyme as determined by SDS polyacrylamide gel. Lane 1 represents unboiled sample and lane 2 represents boiled sample.

Figures 4A-4B is an amino acid alignment of fragments of Stachybotrys chartarum phenol oxidizing enzyme (designated St. ch.) with Myrothecium verracaria bilirubin oxidase (designated biliru oxidas) and LEPTOTHRIX DISCOPHORA manganese oxidizing protein (designated mpf-A).

Figure 5 illustrates the nucleic acid (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence for a phenol oxidizing enzyme obtainable from Stachybotrys chartarum.

Figure 6 illustrates the genomic sequence (SEQ ID NO:3) for a phenol oxidizing enzyme obtainable from Stachybotrys chartarum.

Figure 7 is an amino acid alignment of Stachybotrys phenol oxidizing enzyme SEQ ID NO:2 (bottom line) and Bilirubin oxidase (SEQ ID NO:4).

Figure 8 provides an illustration of the vector pGAPT which was used for the expression of Stachybotrys phenol oxidizing enzyme in Aspergillus. Base 1 to 1134

contains Aspergillus niger glucoamylase gene promoter. Base 1227 to 1485 and 3079 to 3100 contains Aspergillus niger glucoamylase terminator. Aspergillus nidulans pyrG gene was inserted from 1486 to 3078 as a marker for fungal transformation. The rest of the plasmid contains pUC18 sequences for propagation in E. coli. Nucleic acid encoding the Stachybotrys phenol oxidizing enzyme of SEQ ID NO:1 was cloned into the Bgl II and Xba I restriction sites.

Figure 9 shows the nucleic acid sequence of the PCR generated fragment of Stachybotrys described in Example 17 that was expressed in Aspergillus.

Figure 10 is an SDS polyacrylamide gel electrophoresis showing the production of phenol oxidizing enzyme produced by Aspergillus niger var. awamori.

Detailed Description

Definitions

As used herein, the term phenol oxidizing enzyme refers to those enzymes which catalyze redox reactions and are specific for molecular oxygen and hydrogen peroxide as the electron acceptor. When *Stachybotrys* phenol oxidizing enzymes of the present invention are boiled and subjected to SDS gel electrophoresis, three molecular weight species are observed. As used herein, the term "enzyme" encompasses any molecular weight species which alone or in combination with at least one other molecular weight species is able to modify the color associated with a dye or colored compound.

As used herein, *Stachybotrys* refers to any *Stachybotrys* species which produces a phenol oxidizing enzyme capable of modifying the color associated with dyes or colored compounds. The present invention encompasses derivatives of natural isolates of *Stachybotrys*, including progeny and mutants, as long as the derivative is able to produce a phenol oxidizing enzyme capable of modifying the color associated with dye or colored compounds. In a preferred embodiment, the phenol oxidizing enzyme is obtainable from *Stachybotrys* and is purified by the method disclosed in Examples 4 and 5.

As used herein in referring to phenol oxidizing enzymes, the term "obtainable from" means phenol oxidizing enzymes equivalent to those that originate from or are naturally-produced by the particular microbial strain mentioned. To exemplify, phenol oxidizing enzymes obtainable from *Stachybotrys* refer to those phenol oxidizing enzymes which are naturally-produced by *Stachybotrys*. The present invention encompasses phenol oxidizing enzymes identical to those produced by *Stachybotrys* species but which through the use of genetic engineering techniques are produced

by non-*Stachybotrys* organisms transformed with a nucleic acid encoding said phenol oxidizing enzyme. Being equivalent means that the phenol oxidizing enzyme has at least one antigenic group in common with phenol oxidizing enzyme obtainable from S. parvispora MUCL 38996 and/or S. chartarum MUCL 38898 as measured by the Ouchterlony technique in which a positive enzyme exhibits an immunoprecipitation line. Alternatively, being equivalent means that the phenol oxidizing enzyme is encoded by a polynucleotide capable of hybridizing to the polynucleotide having the sequence as shown in SEQ ID NO:1 or SEQ ID NO:3 under conditions of intermediate to maximum stringency. Being equivalent means that the phenol oxidizing enzyme comprises at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identity to the phenol oxidizing enzyme having the amino acid sequence disclosed in SEQ ID NO:2. Percent identity at the nucleic acid level is determined using the FastA program and percent identity at the amino acid level is determined using the TFastA both of which use the method of Pearson and Lipman (PNAS USA, 1988, 85:2444-2448). Alternatively, identity is determined by MegAlign Program from DNAstar (DNASTAR, Inc. Maidson, WI 53715) by Jotun Hein Method (1990, Method in Enzymology, 183: 626-645) with a gap penalty = 11, a gap length penalty = 3 and Pairwise Alignment Parameters Ktuple = 2. The present invention also encompasses mutants, variants and derivatives of the phenol oxidizing enzymes of the present invention as long as the mutant, variant or derivative phenol oxidizing enzyme is able to retain at least one characteristic activity of the naturally occurring phenol oxidizing enzyme.

As used herein, the term 'colored compound' refers to a substance that adds color to textiles or to substances which result in the visual appearance of stains. As defined in Dictionary of Fiber and Textile Technology (Hoechst Celanese Corpora (1990) PO Box 32414, Charlotte NC 28232), a dye is a colored compound that is incorporated into the fiber by chemical reaction, absorption, or dispersion. Exam of dyes include direct Blue dyes, acid Blue dyes, direct red dyes, reactive Blue reactive Black dyes. A catalogue of commonly used textile dyes is found in C Index, 3rd ed. Vol. 1-8. Examples of substances which result in the visual appearance of stains are polyphenols, carotenoids, anthocyanins, tannins, reaction products, etc.

As used herein the phrase "modify the color associated with a dye or colored compound" or "modification of the colored compound" means that the dye or compound is changed through oxidation such that either the color appears modified,

i.e., the color visually appears to be decreased, lessened, decolored, bleached or removed, or the color is not affected but the compound is modified such that dye redeposition is inhibited. The present invention encompasses the modification of the color by any means including, for example, the complete removal of the colored compound from stain on a fabric by any means as well as a reduction of the color intensity or a change in the color of the compound.

The "anti-dye transfer" or "anti-dye redeposition" effect may be a function of the color modification activity of a phenol oxidizing compound, i.e., soluble dyes or colored components are oxidized or bleached and are not able to be redeposited as a color on the fabric, or a function of substrate modification in the absence of color modification such that a dye or colored component becomes water soluble and is rinsed away. The ability of a phenol oxidizing compound used alone or together with an enhancer to oxidize an soluble or dispersed dye or colored compound to a colorless species in a wash solution prevents the color redeposition effect.

As used herein, the term "mutants and variants", when referring to phenol oxidizing enzymes, refers to phenol oxidizing enzymes obtained by alteration of the naturally occurring amino acid sequence and/or structure thereof, such as by alteration of the DNA nucleotide sequence of the structural gene and/or by direct substitution and/or alteration of the amino acid sequence and/or structure of the phenol oxidizing enzyme. The term phenol oxidizing enzyme "derivative" as used herein refers to a portion or fragment of the full-length naturally occurring or variant phenol oxidizing enzyme amino acid sequence that retains at least one activity of the naturally occurring phenol oxidizing enzyme. As used herein, the term "mutants and variants", when referring to microbial strains, refers to cells that are changed from a natural isolate in some form, for example, having altered DNA nucleotide sequence of, for example, the structural gene coding for the phenol oxidizing enzyme; alterations to a natural isolate in order to enhance phenol oxidizing enzyme production; or other changes that effect phenol oxidizing enzyme expression.

The term "enhancer" or "mediator" refers to any compound that is able to modify the color associated with a dye or colored compound in association with a phenol oxidizing enzyme or a compound which increases the oxidative activity of the phenol oxidizing enzyme. The enhancing agent is typically an organic compound.

Phenol oxidizing enzymes

The phenol oxidizing enzymes of the present invention function by catalyzing redox reactions, i.e., the transfer of electrons from an electron donor (usually a

phenolic compound) to molecular oxygen or hydrogen peroxide (which acts as an electron acceptor) which is reduced to water. Examples of such enzymes are laccases (EC 1.10.3.2), bilirubin oxidases (EC 1.3.3.5), phenol oxidases (EC 1.14.18.1), catechol oxidases (EC 1.10.3.1).

The present invention encompasses *Stachybotrys* phenol oxidizing enzymes which are capable of modifying the color associated with a dye or colored compounds and which have at least one antigenic group in common with the phenol oxidizing enzyme naturally-produced by S. parvispora MUCL 38996 and/or the phenol oxidizing enzyme naturally-produced by S. chartarum MUCL 38898. One method for measuring the presence of common antigenic determinants is with the double immunodiffusion tests (Ouchterlony technique) following the protocol set forth in, and under the conditions specified in, Clausen, J. (1988) Immunochemical Technique for the Identification and Estimation of Macromolecules (3rd revised edition) Burdon, R.H., and P.H. van Knippenberg, eds., at page 281 (appendix 11, micro technique) and as interpreted following the protocol described in and under the conditions specified by Clausen, supra, at chapter 6, p143-146. Another method for measuring the presence of common antigenic determinants is by Western blot (Current Protocols in Molecular Biology, Vol.2, John Wiley & Sons, Inc. Section 10.8: Immunoblotting and Immunodetection).

Phenol oxidizing enzyme obtainable from S. *parvispora* MUCL 38996 and produced according to Examples 4 and 5 has an apparent molecular weight of about 38 kilodaltons (kD's) as determined by an SDS-PAGE analysis method and an apparent isoelectric point of lower than 2.8 as defined in Example 6. Phenol oxidizing enzyme obtainable from S. chartarum MUCL 38898 and produced by the method of Examples 4 and 5 has an apparent molecular weight of about 30.9 kilodaltons as determined by an SDS-PAGE analysis method.

The present invention encompasses *Stachybotrys* phenol oxidizing enzymes comprising at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95% identity to the phenol oxidizing enzyme having the amino acid sequence disclosed in SEQ ID NO:2.

Nucleic acid encoding phenol oxidizing enzymes

The present invention encompasses polynucleotides which encode phenol oxidizing enzymes obtainable from *Stachybotrys* species which polynucleotides comprise at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity and at least 95% identity to

the polynucleotide sequence disclosed in SEQ ID NO:1 or SEQ ID NO:3 as long as the enzyme encoded by the polynucleotide is capable of modifying the color associated with dyes or colored compounds. In a preferred embodiment, the phenol oxidizing enzyme has the polynucleotide sequence as shown in SEQ ID NO:1 or as shown in SEQ ID NO:3 or is capable for hybridizing to SEQ ID NO:1 or SEQ ID NO:3 or is complementary thereto. As will be understood by the skilled artisan, due to the degeneracy of the genetic code, a variety of polynucleotides can encode the phenol oxidizing enzyme disclosed in SEQ ID NO: 2. The present invention encompasses all such polynucleotides.

The nucleic acid encoding a phenol oxidizing enzyme may be obtained by standard procedures known in the art from, for example, cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, by PCR, or by the cloning of genomic DNA, or fragments thereof, purified from a desired cell, such as a Stachybotrys species (*See*, for example, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Nucleic acid sequences derived from genomic DNA may contain regulatory regions in addition to coding regions. Whatever the source, the isolated nucleic acid encoding a phenol oxidizing enzyme of the present invention should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis, PCR and column chromatography.

Once nucleic acid fragments are generated, identification of the specific DNA fragment encoding a phenol oxidizing enzyme may be accomplished in a number of ways. For example, a phenol oxidizing enzyme encoding gene of the present invention or its specific RNA, or a fragment thereof, such as a probe or primer, may be isolated and labeled and then used in hybridization assays to detect a generated gene. (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And

Hogness, D., 1975, Proc. Natl. Acad. Sci. USA 72:3961). Those DNA fragments sharing substantial sequence similarity to the probe will hybridize under stringent conditions.

The present invention encompasses phenol oxidizing enzymes obtainable from Stachybotrys species which are identified through nucleic acid hybridization techniques using SEQ ID NO:1 or SEQ ID NO:3 as a probe or primer and screening nucleic acid of either genomic of cDNA origin. Nucleic acid encoding phenol oxidizing enzymes obtainable from Stachybotrys species and having at least 65% identity to SEQ ID NO:1 or SEQ ID NO:3 can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of SEQ ID NO:1 or SEQ ID NO:3. Accordingly, the present invention provides a method for the detection of nucleic acid encoding a phenol oxidizing enzyme encompassed by the present invention which comprises hybridizing part or all of a nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 with Stachybotrys nucleic acid of either genomic or cDNA origin.

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequence disclosed in SEQ ID NO:1 under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA) incorporated herein by reference, and confer a defined "stringency" as explained below.

"Maximum stringency" typically occurs at about Tm-5°C (5°C below the Tm of the probe); "high stringency" at about 5°C to 10°C below Tm; "intermediate stringency" at about 10°C to 20°C below Tm; and "low stringency" at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY).

The process of amplification as carried out in polymerase chain reaction (PCR) technologies is described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from SEQ ID NO:1 or SEQ ID NO:3, preferably about 12 to 30 nucleotides, and more preferably about 25 nucleotides can be used as a probe or PCR primer.

A preferred method of isolating a nucleic acid construct of the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the amino acid sequence of the protein having the amino acid sequence as shown in SEQ ID NO:2. For instance, the PCR may be carried out using the techniques described in US patent No. 4,683,202.

Expression Systems

The present invention provides host cells, expression methods and systems for the production of phenol oxidizing enzymes obtainable from Stachybotrys species in host microorganisms, such as fungus, yeast and bacteria. Once nucleic acid encoding a phenol oxidizing enzyme of the present invention is obtained, recombinant host cells containing the nucleic acid may be constructed using techniques well known in the art. Molecular biology techniques are disclosed in Sambrook et al., Molecular Biology Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). Nucleic acid encoding phenol oxidizing enzymes obtainable from Stachybotrys species and having at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and at least 95% identity to the nucleic acid of SEQ ID NO:1 or SEQ ID NO:2 or which are capable of hybridizing under conditions of intermediate to high stringency or which is complementary to SEQ ID NO:1 or SEQ ID NO:3 is obtained and transformed into a host cell using appropriate vectors. A variety of vectors and transformation and expression cassettes suitable for the cloning,

transformation and expression in fungus, yeast and bacteria are known by those of skill in the art.

Typically, the vector or cassette contains sequences directing transcription and translation of the nucleic acid, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. These control regions may be derived from genes homologous or heterologous to the host as long as the control region selected is able to function in the host cell.

Initiation control regions or promoters, which are useful to drive expression of the phenol oxidizing enzymes in a host cell are known to those skilled in the art. Virtually any promoter capable of driving these phenol oxidizing enzyme is suitable for the present invention. Nucleic acid encoding the phenol oxidizing enzyme is linked operably through initiation codons to selected expression control regions for effective expression of the oxidative or reducing enzymes. Once suitable cassettes are constructed they are used to transform the host cell.

General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using PEG and electroporation. For Aspergillus and Trichoderma, PEG and Calcium mediated protoplast transformation can be used (Finkelstein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156. Electroporation of protoplast is disclosed in Finkelestein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156. Microprojection bombardment on conidia is described in Fungaro et al. (1995) Transformation of Aspergillus nidulans by microprojection bombardment on intact conidia. FEMS Microbiology Letters 125 293-298. Agrobacterium mediated transformation is disclosed in Groot et al. (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi. Nature Biotechnology 16 839-842. For transformation of Saccharomyces, lithium acetate mediated transformation and PEG and calcium mediated protoplast transformation as well as electroporation techniques are known by those of skill in the art.

Host cells which contain the coding sequence for a phenol oxidizing enzyme of the present invention and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

As described herein, the genomic sequence (SEQ ID NO:3) encoding phenol oxidizing enzyme obtainable from Stachybotrys chartarum (MUCL 38898) was isolated and expressed in Aspergillus niger var. awamori and Trichoderma reesei. The cDNA (SEQ ID NO: 1) obtainable from Stachybotrys chartarum (MUCL 38898) was isolated and expressed in Saccharomyces cerevisiae.

Phenol oxidizing enzyme activities

The phenol oxidizing enzymes of the present invention are capable of using a wide variety of different phenolic compounds as electron donors, while being very specific for molecular oxygen or hydrogen peroxide as the electron acceptor.

Depending upon the specific substrate and reaction conditions, e.g., temperature, presence or absence of enhancers, etc., each phenol oxidizing enzyme oxidation reaction will have an optimum pH. For example, the *Stachybotrys* parvispora phenol oxidizing enzyme produced as described in Example 4 has a pH optimum of from about 5.0 to about 7.0, as determined by incubation for 2 minutes at 20 degrees C with ABTS as substrate; a pH optimum of from about 6.0 to about 7.5, as determined by incubation for 2 minutes at 20 degrees C with syringaldizin as substrate; and a pH optimum of from about 7.0 to about 9.0, as determined by incubation for 2 minutes at 20 degrees C with 2,6-dimethoxyphenol as substrate, and which is able to oxidize guiacol.

Phenol oxidizing enzyme obtained from *Stachybotrys* chartarum MUCL 38898, produced as described in Examples 4 and 5 and having the amino acid sequence as shown in SEQ ID NO:2 has a pH optimum of about 8.0 at both 20 and 40 degrees C as determined by incubation with DMP as a substrate and in the presence of a total of 17.2 μg enzyme and a pH optimum of about 5.0 to 7.0 as

determined by incubation with ABTS as a substrate and in the presence of a total of 1.7 μg enzyme.

Applications of polyphenol oxidizing enzymes

As described infra, the phenol oxidizing enzymes obtainable from *Stachybotrys* are capable of oxidizing a wide variety of dyes or colored compounds having different chemical structures, using oxygen or hydrogen peroxide as the electron acceptor. Accordingly phenol oxidizing enzymes of the present invention are used in applications where it is desirable to modify the color associated with dyes or colored compounds, such as in cleaning, for removing the food stains on fabric and anti-dye redeposition; textiles; and paper and pulp applications. A particularly important characteristic of the phenol oxidizing enzymes is their expression of high levels of enzymatic activity, at about 20-40 degrees C, in a broad range of pHs, including a broad range of neutral to alkaline pHs. In particular is their ability to express high levels of enzymatic activity in the pH range of from about 7.0 to about 10.5 in temperatures of about 20- 35 degrees C.

Colored compounds

In the present invention, a variety of colored compounds could be targets for oxidation by phenol oxidizing enzymes of the present invention. For example, in detergent applications, colored substances which may occur as stains on fabrics can be a target. Several types or classes of colored substances which may occur in stains are described below.

Porphyrin derived structures.

Porphyrin structures, often coordinated to a metal, form one class of colored substances which occur in stains. Examples are heme or haematin in blood stain, chlorophyll as the green substance in plants, e.g. grass or spinach. Another example of a metal-free substance is bilirubin, a yellow breakdown product of heme.

Tannins, polyphenols

Tannins are polymerised forms of certain classes of polyphenols. Such polyphenols are catechins, leuantocyanins, etc. (P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd, Edinburgh, 1972, pp.169-198). These substances can be conjugated with simple phenols like e.g. gallic acids. These polyphenolic

substances occur in tea stains, wine stains, banana stains, peach stains, etc. and are notoriously difficult to remove.

Carotenoids.

Carotenoids are the coloured substances which occur in tomato (lycopene, red), mango (carotene, orange-yellow) (G.E. Bartley et al., The Plant Cell (1995), Vol 7, 1027-1038). They occur in food stains (tomato) which are also notoriously difficult to remove, especially on colored fabrics, when the use of chemical bleaching agents is not advised.

Anthocyanins.

These substance are the highly colored molecules which occur in many fruits and flowers (P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd, Edinburgh, 1972, 135-169). Typical examples, relevant for stains, are berries, but also wine. Anthocyanins have a high diversity in glycosidation patterns.

Maillard reaction products

Upon heating of mixtures of carbohydrate molecules in the presence of protein/peptide structures, a typical yellow/brown colored substance arises. These substances occur for example in cooking oil and are difficult to remove from fabrics.

For the prevention of dye transfer from a colored piece of fabric to other garments during the wash, it is desirable to specifically bleach the dye molecules in the wash solution. A variety of types of fabric dyes are desirable targets for the oxidation process: e.g. sulphur dyes, vat dyes, direct dye, reactive dyes and azoic dyes.

Enhancers

A phenol oxidizing enzyme of the present invention can act to modify the color associated with dyes or colored compounds in the presence or absence of enhancers depending upon the characteristics of the compound. If a compound is able to act as a direct substrate for the phenol oxidizing enzyme, the phenol oxidizing enzyme can modify the color associated with a dye or colored compound in the absence of an enhancer, although an enhancer may still be preferred for optimum phenol oxidizing enzyme activity. For other colored compounds unable to act as a direct substrate for the phenol oxidizing enzyme or not directly accessible to

the phenol oxidizing enzyme, an enhancer is required for optimum phenol oxidizing enzyme activity and modification of the color.

Enhancers are described in for example WO 95/01426 published 12 January 1995; WO 96/06930, published 7 March 1996; and WO 97/11217 published 27 March 1997. Enhancers include but are not limited to phenothiazine-10-propionic acid (PPT), 10-methylphenothiazine (MPT), phenoxazine-10-propionic acid (PPO), 10-methylphenoxazine (MPO), 10-ethylphenothiazine-4-carboxylic acid (EPC) acetosyringone, syringaldehyde, methylsyringate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate (ABTS) and 4-Hydroxy-4-biphenyl-carboxylic acid or derivatives thereof.

Cultures

The present invention encompasses *Stachybotrys* strains and natural isolates, and derivatives of such strains and isolates, such as strains of the species *S.* parvispora, including, in particular, *S.*parvispora var. hughes MUCL 38996; strains of the species *S.* chartarum including, in particular, *Stachybotrys* chartarum MUCL 38898; S. parvispora MUCL 9485; S. chartarum MUCL 30782; S. kampalensis MUCL 39090; S. theobromae MUCL 39293; and strains of the species S. bisbyi, S. cylindrospora, S. dichroa, S. oenanthes and S. nilagerica which produce phenol oxidizing enzymes of the present invention.

The present invention provides substantially biologically-pure cultures of novel strains of the genus *Stachybotrys*, and, in particular substantially biologically-pure cultures of the strains *S.* parvispora MUCL 38996 and *S.* chartarum MUCL 38898 from which phenol oxidizing enzymes can be purified.

Purification

The phenol oxidizing enzymes of the present invention may be produced by cultivation of phenol oxidizing enzyme-producing *Stachybotrys* strains (such as S. parvispora MUCL 38996, S. chartarum MUCL 38898) under aerobic conditions in nutrient medium containing assimiable carbon and nitrogen together with other essential nutrient(s). The medium can be composed in accordance with principles well-known in the art.

During cultivation, the phenol oxidizing enzyme-producing strains secrete phenol oxidizing enzyme extracellularly. This permits the isolation and purification

(recovery) of the phenol oxidizing enzyme to be achieved by, for example, separation of cell mass from a culture broth (e.g. by filtration or centrifugation). The resulting cell-free culture broth can be used as such or, if desired, may first be concentrated (e.g. by evaporation or ultrafiltration). If desired, the phenol oxidizing enzyme can then be separated from the cell-free broth and purified to the desired degree by conventional methods, e.g. by column chromatography, or even crystallized.

The phenol oxidizing enzymes of the present invention may be isolated and purified from the culture broth into which they are extracellularly secreted by concentration of the supernatant of the host culture, followed by ammonium sulfate fractionation and gel permeation chromatography.

The phenol oxidizing enzymes of the present invention may be formulated and utilized according to their intended application. In this respect, if being used in a detergent composition, the phenol oxidizing enzyme may be formulated, directly from the fermentation broth, as a coated solid using the procedure described in United States Letters Patent No. 4,689,297. Furthermore, if desired, the phenol oxidizing enzyme may be formulated in a liquid form with a suitable carrier. The phenol oxidizing enzyme may also be immobilized, if desired.

The present invention also encompasses expression vectors and recombinant host cells comprising a *Stachybotrys* phenol oxidizing enzyme of the present invention and the subsequent purification of the phenol oxidizing enzyme from the recombinant host cell.

Detergent Compositions

A *Stachybotrys* phenol oxidizing enzyme of the present invention may be used in detergent or cleaning compositions. Such compositions may comprise, in addition to the phenol oxidizing enzyme, conventional detergent ingredients such as surfactants, builders and further enzymes such as, for example, proteases, amylases, lipases, cutinases, cellulases or peroxidases. Other ingredients include enhancers, stabilizing agents, bactericides, optical brighteners and perfumes. The detergent compositions may take any suitable physical form, such as a powder, an aqueous or non aqueous liquid, a paste or a gel. Examples of detergent

compositions are given in WO 95/01426, published 12 January 1995 and WO 96/06930 published 7 March 1996.

Having thus described the phenol oxidizing enzymes of the present invention, the following examples are now presented for the purposes of illustration and are neither meant to be, nor should they be, read as being restrictive. Dilutions, quantities, etc. which are expressed herein in terms of percentages are, unless otherwise specified, percentages given in terms of per cent weight per volume (w/v). As used herein, dilutions, quantities, etc., which are expressed in terms of % (v/v), refer to percentage in terms of volume per volume. Temperatures referred to herein are given in degrees centigrade (C).

Example 1

Isolation and Identification of *Stachybotrys* parvispora var. hughes Strain

A new strain of the species *Stachybotrys* parvispora var. hughes was isolated from soil samples on an agar-agar nutrient medium and selected by its production of an enzyme having oxidase activity.

The new strain was individually cultured on corn meal agar (DIFCO) at 25 degrees C for a period of three weeks.

The new strain of S. parvispora was identified by its slow growth in corn meal agar at 25 degrees C, being less than 4 cm in three weeks, its formation of conidia and the morphological characteristics of the formed conidia.

After growth for three days on corn meal agar at 25 degrees C, microscopic observation revealed that the cells of the new strain of S. parvispora have the form of conidia of 5.25 x 3.75-4.5 mm in size which are coarsely roughened and are gathered in a dark olive gray mucilaginous drop, borne from phialides 9-11 x 3.5-4.5 mm clustered in verticille. Conidiophores are smooth-walled, up to 200 mm long (see Jong, S.C and E.E. Davis, Mycotaxon 3:409-485.).

The new strain of S. parvispora so identified was deposited under the provisions of the Treaty of Budapest in the Belgian Coordinated Collections of Microorganisms, Mycothäque de l'Universitä Catholique de Louvain (MUCL), Place Croix du Sud 3, Louvain-La-Neuve, Belgium B-1348 on 5 December 1995 and given accession number MUCL 38996.

Example 2

Isolation and Identification of *Stachybotrys* chartarum Strain

A new strain of the species *Stachybotrys* chartarum (formerly named *Stachybotrys* atra var. corda) was isolated from soil samples on an agar-agar nutrient medium and selected by its production of an enzyme having oxidase activity.

The new strain was individually cultured on corn meal agar (DIFCO) at 25 degrees C for a period of three weeks.

The new strain S. chartarum was identified by its rapid growth on corn meal agar at 25 degrees C, being more than 4 cm in three weeks, its formation of conidia and the morphological characteristics of the formed conidia.

After growth for three days on corn meal agar at 25 degrees C, microscopic observation revealed that the cells of the new strain of S. chartarum have the form of conidia of 8-11 x 5-10 mm in size which are coarsely roughened and are gathered in a dark olive gray mucilaginous drop, borne from phialides 10-13 x 4-6 mm clustered in verticille. Conidiophores are smooth-walled, up to 1000 mm long (see Jong, S.C and E.E. Davis, Mycotaxon 3:409-485).

The new strain of S. chartarum so identified was deposited under the provisions of the Treaty of Budapest in the Belgian Coordinated Collections of Microorganisms, Mycothäque de l'Universitä Catholique de Louvain (MUCL), Place Croix du Sud 3, Louvain-La-Neuve, Belgium B-1348 on 5 December 1995 and given accession number MUCL 38898.

Example 3

Preparation of Conidial Stock Suspension for Inoculation

Stachybotrys parvispora MUCL 38996, obtained as described above in Example 1, was isolated on PDA (potato dextrose agar) plates (DIFCO).

One colony was suspended in 5 ml of 0.9% (w/v) NaCl, containing about 30 sterile glass beads (diameter 5mm). The suspension was thoroughly agitated in a vortex mixer (BENDER & HOBEIN AG), until complete homogenization of the mycelium was obtained (full speed for approximately 15-20 minutes) . Several dilutions (ranging from 10^{-5} to 10^{-7}) of this homogenate were then plated on respective sterile PDA plates and incubated at 30 degrees C for about 5 weeks to allow formation of conidia (dark-brownish in color).

Three plates, each containing approximately 50 isolated sporulated colonies (as evidenced by their dark-brownish color) were then spread with 5 ml of 0.9% (w/v) NaCl and scraped with a glass rod to suspend the conidia. The resulting suspensions were pooled and filtered using Miracloth (CALBIOCHEM) membrane in order to remove the remaining mycelium. The result were conidial stock suspensions.

The titer (measured in terms of colony forming units (cfu) per ml) of the resulting suspension was then determined by plating dilutions [in 0.9% (w/v) NaCl] on PDA plates. The titers of the resulting conidial stock suspensions ranged from 10^6 to 10^7 cfu/ml.

Example 4

Production of Phenol Oxidizing Enzyme

Production of Enzyme from *Stachybotrys* parvispora

A twenty liter fermentor containing glucose and potato extract was prepared by boiling 4.5 kilograms of peeled and diced potatoes for 30 minutes in 15 liters of water (milli-Q quality), filtering the resulting suspension through hydrophilic cotton gauze (STELLA), collecting the resulting filtrate and then supplementing the collected filtrate with 300 grams of glucose. The glucose supplemented filtrate was then placed in the fermentor and sterilized for 30 minutes at 120°C. The sterilized supplemented filtrate had a pH of 5.8.

The twenty liter fermentor was then inoculated with 15 ml of the conidial stock suspension, obtained as described above in Example 3, and fermentation was conducted for 144 hours at 37 degrees C.

Fermentation was performed under a constant air flow of 4.5 liters/minute and a constant agitation of 100 RPM (revolutions per minute) (diameter 13 cm) without pH control.

An approximately 50 ml sample of the culture (fermentation) broth was then withdrawn from the fermentor and centrifuged at 12,000 g for 5 minutes. The supernatant was then removed from the pellet.

The presence of phenol oxidizing enzyme activity in the supernatant was then measured using the following standard assay procedure, based on the oxidation of ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)] by oxygen : a final

reaction volume of 1 ml containing Tris [Tris(hydroxymethyl)-aminomethane]/HCl 200 mM (pH 7.0), 0.9 mM ABTS (Diammonium salt from SIGMA) and an appropriate amount of the preparation to be assayed (which, in this example, is the supernatant diluted with water as described below) was prepared. The assay reaction was started by the addition of the preparation to be assayed (which in this example is the supernatant dilution) to form the final 1 ml reaction volume. The greenish-blue color produced by the oxidation of ABTS was then continually measured by recording the optical density (OD) at 420 nm during two minutes, using a spectrophotometer (Ultraspec Plus from Pharmacia). The rate of increase of the optical density per minute (ΔOD/minute) was then calculated from the linear part of the curve during 1 minute.

The appropriate amount of the (enzyme) preparation submitted to this standard assay, was adjusted by dilution with water in order to obtain a ΔOD/minute ranging from 0.2 to 1.0 during the assay.

As used herein, one standard ABTS enzyme unit (hereinafter referred to as one enzyme unit or EU) is defined as the amount of enzyme that produces an increase of one OD^{420} per minute, under these specific conditions.

In this manner, an enzyme activity of 30 EU/ml of culture supernatant was measured.

<u>*Stachybotrys* chartarum phenol oxidizing enzyme production</u>

Stachybotrys chartarum was grown on PDA plates (Difco) for about 5 - 10 days. A portion of the plate culture (about 3/4 x 3/4 inch) was used to inoculate 100 ml of PDB (potato dextrose broth) in 500-ml shake flask. The flask was incubated at 26 - 28 degrees C, 150 rpm, for 3 - 5 days until good growth was obtained.

The broth culture was then inoculated into 1 L of PDB in a 2.8-L shake flask. The flask was incubated at 26 - 28 degrees C, 150 rpm, for 2 - 4 days until good growth was obtained.

A 10-L fermentor containing a production medium was prepared (containing in grams/liter the following components: glucose 15; lecithin1.51; t-aconitic acid 1.73; KH_2PO_4 3; $MgSO_4.7H_2O$ 0.8; $CaCl_2.2H_2O$ 0.1; ammonium tartrate 1.2; soy peptone 5; Staley 7359; benzyl alcohol 1; tween 20 1; nitrilotriacetic acid 0.15; $MnSO_4.7H_2O$ 0.05; NaCl 0.1; $FeSO_4.7H_2O$ 0.01; $CoSO_4$ 0.01; $CaCl_2.2H_2O$ 0.01; $ZnSO_4.7H_2O$ 0.01;

$CuSO_4$ 0.001; $ALK(SO_4)2.12H_2O$ 0.001; H_3BO_3 0.001; $NaMoO_4.2H_2O$ 0.001). The fermentor was then inoculated with the 1-L broth culture, and fermentation was conducted at 28 degrees C for 60 hours, under a constant air flow of 5.0 liters/minute and a constant agitation of 120 RPM. The pH was maintained at 6.0.

The presence of phenol oxidizing enzyme activity in the supernatant was measured using the following assay procedure, based on the oxidation of ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)) by oxygen. ABTS (SIGMA, 0.2 ml, 4.5 mM H_2O) and NaOAc (1.5ml, 120mM in H_2O,pH 5.0) were mixed in a cuvette. The reaction was started by addition of an appropriate amount of the preparation to be measured (which in this example is the supernatant dilution) to form a final solution of 1.8 ml. The color produced by the oxidation of ABTS was then measured every 2 seconds for total period of 14 seconds by recording the optical density (OD) at 420 nm, using a spectrophotometer. One ABTS unit (one enzyme unit or EACU) in this example is defined as the change in OD measured at 420 per minute/2 (given no dilution to the sample). In this manner a phenol oxidizing enzyme activity of 3.5 EACU/ml of culture supernatant was measured.

Example 5

Purification of the Enzyme

The remaining Stachybotrys parvispora culture broth, obtained as described above in Example 4, was then withdrawn from the fermentor and centrifuged for 15 minutes at 4,500 g. Stachybotrys chartarum is purified in a similar fashion.

The resulting supernatant was then removed from the pellet and concentrated to 0.6 liters by ultrafiltration using a Amicon ultrafiltration unit equipped with a YM10 membrane having a 10 kD cutoff.

A volume of 1.4 liters of acetone was added to the concentrate and mixed therewith. The resulting mixture was then incubated for two hours at 20-25 degrees C.

Following incubation, the mixture was centrifuged for 30 minutes at 10,000 g and the resulting pellet was removed from the supernatant. The pellet was then resuspended in a final volume of 800 ml of water.

The resulting suspension was then submitted to ammonium sulfate fractionation as follows : crystalline ammonium sulfate (JANSSEN) was added to the

suspension to 40% saturation and the mixture incubated at 4 degrees C for 16 hours with gentle magnetic stirring. The mixture was then centrifuged at 10,000 g for 30 minutes and the supernatant removed from the centrifugation pellet for further use. Ammonium sulfate (JANSSEN) was then added to the supernatant to reach 80% saturation, and the mixture incubated at 4 degrees C for 16 hours with gentle magnetic stirring. The suspension was then centrifuged for 30 minutes at 10,000 g and the resulting pellet was removed from the supernatant. The pellet was then resuspended in 15 ml of water and concentrated to 6 ml by ultrafiltration using a CENTRIPREP 3000 (AMICON).

The phenol oxidizing enzyme activity of the suspension was then measured using the standard assay procedure, based on the oxidation of ABTS by oxygen, as was described above in Example 4 (but with the exception that the preparation being assayed is the resuspended concentration and not the supernatant dilutions). The phenol oxidizing enzyme activity so measured was 5200 EU/ml.

The enzyme was then further purified by gel permeation chromatography. In this regard, a column containing 850 ml of SEPHACRYL S400 HIGH RESOLUTION (PHARMACIA) was equilibrated with a buffer containing 50 mM KH_2PO_4/K_2HPO_4 (pH = 7.0) and then loaded with the remainder of the 6 ml suspension described above, and eluted with the buffer containing 50 mM KH_2PO_4/K_2HPO_4 (pH = 7.0), at a flow rate of 1 ml/minute. Respective fractions were then obtained.

The respective fractions containing the highest phenol oxidizing enzyme activities were pooled together, providing a 60 ml suspension containing the purified phenol oxidizing enzyme.

The phenol oxidizing enzyme activity of the suspension was then measured using the standard assay procedure, based on the oxidation of ABTS by oxygen, as was described above in Example 4. The enzyme activity so measured was 390 EU/ml.

This preparation was then used for further characterization of the enzyme, as will be described at length below.

Example 6

Determination of Isoelectric Point of S. parvispora Phenol Oxidizing Enzyme

The isoelectric point (pI) of the enzyme produced by S. parvispora MUCL 38996, was then determined from the purified enzyme, obtained as described above in Example 5.

This determination was effectuated by isoelectric focalization in polyacrylamide gels, by employing Pharmacia DryIEF Gels, that had been rehydrated with 2 ml of an ampholine solution (1 volume Pharmacia 8-10.5% (w/v) ampholine added to 15 volumes of deionized water), following the protocol recommended by the supplier.

The purified enzyme, obtained as described above in Example 5, was submitted to an isoelectric focusing gel (IEF 3-9 from PHARMACIA), as described in the PHARMACIA Technical File IEF No 100.

The following PHARMACIA reference markers were used in this isoelectric focusing: pepsinogen (2.8), amyloglucosidase (3.5), methyl red (3.75), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), b-lactoglobulin A (5.2), bovine carbonic anhydrase B (5.85) and human carbonic anhydrase B (6.55).

The samples to be submitted to isoelectric focusing were prepared, as described in the PHARMACIA Technical File IEF No 100.

After focusing, gels were stained with Coomassie Blue following the protocol detailed in the Separation Technique File No. 101 (publication 18-1018-20, Pharmacia LKB Biotechnology).

Using this technique, the apparent isoelectric point of the phenol oxidizing enzyme secreted from S. parvispora MUCL 38996 was determined to be lower than 2.8.

Example 7

Determination of pH optimum for S. parvispora and S. chartarum phenol oxidizing enzyme

Thirteen 100 ml buffer samples, each containing 50 mM Tris, 50 mM citric acid and 50 mM Na_2HPO_4 were prepared.

The thirteen buffer samples were then adjusted to the respective pHs noted below in Table 1A with either HCl or NaOH, as applicable, so that one of the buffer samples possessed each of the pH values noted below in Table 1A.

Three 0.9 ml samples were then taken from each of the thirteen buffer samples. In this manner, three groups (a first group, a second group and a third group) of thirteen samples each were provided, so that each group possessed a respective sample of each of the thirteen pH buffer samples.

Respective substrates were then added to respective mixtures as follows : 0.9 mM ABTS was added to the thirteen mixtures of the first group; 50 μM DMP (2,6-dimethoxyphenol) (FLUKA) was added to the thirteen mixtures of the second group; and 1mM syringaldizin (SIGMA) was added to the thirteen mixtures of the third group.

The respective reactions were started by the addition of 2 EU of purified phenol oxidizing enzyme from S. parvispora MUCL 38996, obtained as described above in Example 5.

The final volume of each of the samples assayed was 1 ml.

The assays on each of the thirty-nine samples (adjusted to the pH values noted below in Table 1A) were performed at approximately 20 degrees C with an incubation time of 2 minutes following the protocol set forth above in Example 4.

The optical density was recorded during 2 minutes (Ultraspec Plus from Pharmacia) at the following wavelengths : 420 nm for the samples of the first group (having ABTS), 468 nm for the samples of the second group (having DMP) and 526 nm (for the samples of the third group (having syringaldazine).

The rate of increase of the optical density (DOD/min) was calculated from the linear part of the curves during one minute, as described at length above in Example 4.

The assay results are summarized below in Table 1A.

Table 1A

Activity (ΔOD/minute/ml) for S. parvispora enzyme

pH	ABTS	Syringaldazin	2,6 dimethoxyphenol
4.0	0.76	0.00	0.21
4.5	0.89	0.00	0.21
5.0	2.04	0.00	0.32

pH	ABTS	Syringaldazin	2,6 dimethoxyphenol
5.5	2.0	0.25	0.43
6.0	2.11	1.27	0.61
6.5	2.14	1.61	0.91
7.0	2.04	1.75	1.59
7.5	1.54	1.43	2.52
8.0	0.93	0.92	3.52
8.5	0.42	0.87	3.18
9.0	0.11	0.68	1.41
9.5	0.03	0.03	0.08
10.0	0.00	0.00	0.08

In a similar manner, the pH profile for S. chartarum phenol oxidizing enzyme was obtained. Instead of 50μM DMP, 5mM DMP was used. The amount of enzyme used per ABTS assay was 1.7 μg enzyme in a total of 0.9 ml assay. The amount of enzyme used per DMP assay was 17.2 μg in a total of 0.9 ml assay. The results are given in Table IB

Table IB
Determination pH optimum Stachybotrys Charatum enzyme

Activity (ΔOD/ minute/ml)

pH	ABTS (20 °C)	ABTS (40 °C)	DMP (20 °C)	DMP (40 °C)
4	2.60	1.72	0.01	0.03
4.5	3.26	1.73	0.01	0.03
5	3.83	1.55	0.01	0.03
5.5	4.37	1.57	0.02	0.04
6	4.25	1.54	0.04	0.09
6.5	4.45	1.50	0.08	0.18
7	3.65	2.70	0.21	0.33
7.5	3.01	3.31	0.47	0.63
8	2.16	3.41	0.62	0.84
8.5	1.15	2.85	0.46	0.81
9	0.42	1.07	0.29	0.60
9.5	0.19	0.45	0.20	0.58
10	0.10	0.19	0.01	0.33
10.5	0.04	0.02	0.04	0.06
11	0.00	0.00	0.07	0.04
11.5	0.00	0.00	0.00	0.01

pH	ABTS (20 °C)	ABTS (40 °C)	DMP (20 °C)	DMP (40 °C)
12	0.00	0.00	0.00	0.00

DMP = 2,6 dimethoxyphenol
Assay was carried out at 20 C and 40 C

The above protocol for Stachybotrys parvispora was repeated with the exceptions that all of the buffer samples were adjusted to a pH of 7.0 and that the substrates employed were 5mM of either s-dianizidine (SIGMA), 3,4-dimethoxyphenol (FLUKA), 3,4-dimethoxyaniline (FLUKA), 3-methoxy phenol (FLUKA) and veratrylic alcohol (SIGMA).

With the exception of veratrylic alcohol, color formation was observed qualitatively from each of these other substrates.

Example 8

Comparison with Bilirubin Oxidase

pH profile of DBI Bleaching

14 reaction mixtures (1 ml final volume) were prepared containing 50 mM Tris, 50 mM citric acid and 50 mM Na2HPO4, with two of each of the said reaction mixtures being adjusted to each of the respective pHs indicated below in Table 2 with either HCl or NaOH, and the substrate, Direct Blue No. 1 (herein referred to as DBI, also known as Chicago Sky Blue 6B) from (SIGMA) was added thereto in a quantity necessary for obtaining an initial Optical Density (OD) of 1.0 (620 nm).

The respective reactions were started by the addition to the respective reaction mixtures of 4.5 EU of phenol oxidizing enzyme from either S. parvispora MUCL 38996 obtained as described above in Example 5, or the bilirubin oxidase from Myrothecium verrucaria (purchased from SIGMA).

The final volume of each of the samples assayed was 1 ml.

The assays on each of the samples were performed at approximately 20 degrees C with an incubation time of 2 minutes following the protocol set forth above in Example 4.

The optical density was recorded during 2 minutes (Ultraspec Plus from Pharmacia), at a wavelength of 620 nm. The rate of decrease of the optical density (-ΔOD/min) was calculated from the linear part of the curves.

The assay results are summarized below in Table 2.

Table 2

Activity (-ΔOD/minute/ml)		
pH	*Stachybotrys*	Myrothecium
4.0	2.65	4.10
5.0	2.65	4.20
6.0	3.85	4.50
7.0	4.95	4.75
8.0	6.95	3.60
9.0	8.90	1.45
10.0	5.85	1.10

Oxidation of quiacol

Reaction mixtures (1 ml final volume) were prepared containing 200 tmM Tris/HCl (pH 7.0) and 5 mM quiacol (2-methoxyphenol) (MERCK) as substrate.

The reactions were started by the addition of 5 EU of phenol oxidizing enzyme from S. parvispora MUCL 38996, obtained as described above in Example 5, or by the addition of 5 EU of the bilirubin oxidase from Myrothecium verrucaria (purchased from SIGMA).

The final volume of each of the samples assayed was 1 ml.
The assays on each of the samples were performed at approximately 20 degrees C with an incubation time of 2 minutes following the protocol set forth above in Example 4.

The optical density was recorded during 2 minutes (Ultraspec Plus from PHARMACIA), at a wavelength of 440 nm. The rate of increase of the optical density (ΔOD/min) was calculated from the linear part of the curves.

With the phenol oxidizing enzyme from *Stachybotrys* parvispora MUCL 38996, an increase of OD was recorded (0.05 ΔOD/min). However, no activity was detectable with the bilirubin oxidase from Myrothecium verrucaria.

Example 9

Bleaching of Various Dyes

The substrate specificity of the phenol oxidizing enzyme from S. parvispora MUCL 38996 was studied versus a number of dyes. The reaction mixtures (1 ml final volume) contained 200 mM Tris/HCl (pH 7.0) and the respective dyes listed below in

Table 3, the concentration of which dyes were adjusted by dilution with water, so that an optical density of 1.0 (at the wavelengths listed below in Table 3) was measured therefor. The reactive and dye nomenclature is in accordance with the color index.

The bleaching reactions were started by the addition of phenol oxidizing enzyme of S. parvispora MUCL 38996, obtained as described above in Example 5. The amount of phenol oxidizing enzyme was adjusted by dilution with water in order to measure a decrease in OD (at the wavelengths listed in Table 3) in the range of 0.05 to 0.25 - ΔOD/minute, in order to obtain a linear curve.

The final volume of each of the samples assayed was 1 ml.

The assays on each of the samples were performed at approximately 20°C with an incubation time of 2 minutes following the protocol set forth above in Example 4.

The optical density was recorded during 2 minutes, at the wavelength indicated in Table 3 (Ultraspec Plus from Pharmacia). The rate of decrease of the optical density (-ΔOD/min) was calculated from the linear part of the curve, and multiplied by the enzyme dilution in order to express the final bleaching rate in - ΔOD/minute/ml of enzyme solution obtained as described above in Example 5.

The results are summarized below in Table 3.

In a separate experiment, the rate of oxygen consumption was measured with each of the dyes, in a magnetically stirred chamber equipped with a Clark electrode (oxygraph K-IC from Gilson). The oxygraph chamber contained, in a final volume of 2 ml, 200 mM Tris/HCl (pH 7.0), 5 mM of each of the dyes, and 100 ml (39 EU) of phenol oxidizing enzyme from S. parvispora MUCL 38996, obtained as described above in Example 5. The reactions were started by the addition of the enzyme, and the dissolved oxygen concentration was recorded during 5 minutes. The slope of the curves were determined from their linear parts.

The results of this experiment are also summarized below in Table 3.

Table 3

Dye	Wavelength (nm)	Bleaching Rate -ΔOD/min/ml	Oxygen Consumption - ΔOD/min/ml
Direct Blue 14 (SIGMA)	584	2.5	6.5
Direct Blue 1 (SIGMA)	620	2.0	6.0
Direct blue 53 (FLUKA)	590	4.2	4.6
Direct Blue 98 (ZENECA)	580	0.4	N.D.

Dye	Wavelength (nm)	Bleaching Rate -ΔOD/min/ml	Oxygen Consumption - ΔOD/min/ml
Acid Blue 113 (ALDRICH)	539	0.6	N.D.
Direct Red 28 (SIGMA)	480	0.2	0.6
Direct Red 21 (FLUKA)	494	0.3	1.4
Direct Red 79 (ZENECA)	509	0.2	N.D.
Reactive Blue Cibacron GN_E (CIBA-GEIGY)	622	16.4	4.0
Reactive Blue Cibacron C-R (CIBA-GEIGY)	610	7.8	4.3
Reactive Blue 160 (ZENECA)	617	2.7	N.D.
Direct Blue 71 (ZENECA)	507	0.0	1.3
Reactive Black 5 (SANDOZ)	600	0.0	2.5
Malvin (ROTH)	526	2.6	N.D.

N.D. refers to Not Determined

These results demonstrate that the Stachybotrys phenol oxidizing enzyme is able to oxidize and bleach a variety of dyes exhibiting different chemical structures, using oxygen as the electron acceptor, and in the absence of mediators.

Two dyes (reactive black 5 and direct blue 71) are oxidized by the Stachybotrys phenol oxidizing enzyme, but no bleaching reaction can be observed. However, anti-dye transfer tests (see Example 12 below), show that the transfer of reactive black 5 can indeed be prevented. Thus, even though the dye is not directly bleached by the phenol oxidizing enzyme, it seems to be modified in such a way that the transfer is inhibited.

The results summarized in Table 3 also show that natural dyes of the anthocyanin type, like malvin, can be efficiently bleached by the phenol oxidizing enzyme, which demonstrates its efficiency for removing stains containing such type of dyes (such as fruit-wine, etc.)

Example 10

Immunological Properties

Purified phenol oxidizing enzyme from S. parvispora MUCL 38996, obtained as described above in Example 5, was diluted twice with water, and 0.5 ml of this solution was mixed with 0.5 ml of complete Freund adjuvant, and subcutaneously injected into a rabbit as described in Antibodies (1988) Cold Spring Harbor Laboratory, Harlow and Lane eds, at page 105. This immunization procedure was

repeated three more times (giving four times total), allowing a 2 week time interval between each injection.

Two weeks after the fourth injection, the antisera were collected as described in Antibodies (1988) supra, at page 119.

Double immunodiffusion tests (Ouchterlony technique) were then performed following the protocol set forth in, and under the conditions specified in, Clausen, J. (1988) Immunochemical Technique for the Identification and Estimation of Macromolecules (3rd revised edition) Burdon, R.H., and P.H. van Knippenberg, eds., at page 281 (appendix 11, micro technique).

Four respective microscope slides (25 mm x 75 mm x 1 mm) were prepared, each being covered with 2.5 ml of melted diffusion medium, composed of 1.7 % (w/v) agar (Agar granulated from Difco no 0145-17-0), and 0.9 % (w/v) NaCl, following the technique described in Clausen, supra (at appendix 10, § 10.1: microtechnique). Five wells were then made in the agar of each slide using a template with a sucking device (as also described in Clausen, supra, at appendix 10, § 10.1.1.1). The five wells (one in the center and four encircling the center well) made in the slides each had respective diameters of 3 mm, with a distance between the wells (center to center) of 8 mm being provided.

S. chartarum MUCL 38898 (obtained as described above in Example 2) was isolated on Malt Extracted Plates (ME from DIFCO). One colony thereof was then suspended in 5 ml of 0.9% (w/v) NaCl containing about 30 sterile glass beads (diameter 5 mm). The suspension was thoroughly agitated with a vortex mixer until complete homogenization of the mycelium was obtained. 30 grams of TSB (Trypticase Soy Broth from BECTON DICKINSON) powder were dissolved in 1 liter of water and sterilization performed by heating at 120 degrees C for 30 minutes. Respective 500 ml quantities of the sterilized culture medium were then added to two polypropylene shaking flasks (volume 2 liters). The flasks were then inoculated with respective 1 ml samples of the mycelium suspension and run for 96 hours under constant agitation (100 RPM with 1 inch eccentricity) at 37°C.

After fermentation, the culture medium from the respective shaking flasks were centrifuged at 10000 g for 15 minutes. The resulting supernatants were then removed and each was concentrated 20 times by acetone precipitation (1 volume supernatant/ 3 volumes acetone). The mixtures were then incubated at 4°C under magnetic stirring for 45 minutes. The resulting suspensions were then again centrifuged at 10000 g for 15 minutes and the resulting pellets removed therefrom.

The removed pellets were then resuspended in 50 ml water (Milli-Q quality). A phenol oxidizing enzyme activity of 0.5 U ABTS was measured on ABTS. The resulting enzymatic solutions were then used for the immunological tests.

Respective dilutions of 2X (having 1 volume of enzyme and 1 volume of diluant); 4X (having 1 volume of enzyme sample and 3 volumes of diluant) and 8X (having 1 volume of enzyme sample and 7 volumes of diluant) were prepared using 0.9 % (w/v) NaCl as diluant and of 0.6 EU enzyme samples of S. parvispora MUCL 38996 phenol oxidizing enzyme (obtained as described above in Example 5) the S. chartarum MUCL 38898 phenol oxidizing enzyme (obtained as described below) and the bilirubin oxidase of M. verrucaria (SIGMA).

A constant volume of 10 m1 of the respective samples (dilutions) to be tested were then loaded into the respective four encircling wells (as described below) and the antiserum raised against the phenol oxidizing enzyme activity obtained from S. parvispora MUCL 38996 was loaded in the center well (as is also described below). The slides were then incubated during 18 hours at 37°C, before being examined on a black background using a slit lamp. The four slides so prepared contained the following samples:

Slide	Well 1	Well 2	Well 3	Well 4	Center Well
A	1	2	3	4	Antiserum
B	5	6	7	8	Antiserum
C	9	10	11	12	Antiserum
D	1	5	9	13	Antiserum

Sample 1 is an undiluted sample of S. parvispora enzyme.
Sample 2 is a 2X dilution of S. parvispora enzyme.
Sample 3 is a 4X dilution of S. parvispora enzyme.
Sample 4 is an 8X dilution of S. parvispora enzyme.
Sample 5 is an undiluted sample of S. chartarum enzyme.
Sample 6 is a 2X dilution of S. chartarum enzyme.
Sample 7 is a 4X dilution of S. chartarum enzyme.
Sample 8 is an 8X dilution of S. chartarum enzyme.
Sample 9 is an undiluted sample of M. verrucaria bilirubin oxidase.
Sample 10 is a 2X dilution of M. verrucaria bilirubin oxidase.
Sample 11 is a 4X dilution of M. verrucaria bilirubin oxidase.

Sample 12 is an 8X dilution of M. verrucaria bilirubin oxidase.
Sample 13 is a 1/1 (v/v) mixture of undiluted samples of S.parvispora phenol oxidizing enzyme and M. verrucaria bilirubin oxidase.

Interpretations of the precipitation reactions resulting from this test were then performed following the protocol described in and under the conditions specified by Clausen, supra, at chapter 6, p143-146.

The results of Slide A (which contained the various dilutions of the S. parvispora phenol oxidizing enzyme) showed a clear precipitation arc (or immunoprecipitation line) of the type designated Type I which has been identified as being typical of complete identity (see Clausen, supra, at pages 144-146, § 6.1.2.1). This was expected in that the antiserum was raised against the S. parvispora phenol oxidizing enzyme.

The results of Slide B (which involved the same test being performed using the same protocol and under the same conditions as described at length above in this example with equivalent quantities (EU) of the *Stachybotrys* chartarum MUCL no 38898 phenol oxidizing enzyme) showed a clear precipitation arc of the type designated TYPE I, which has been identified as being typical of complete identity (see Clausen, supra, at pages 144-146, § 6.1.2.1).

The results of Slide C (which involve the same test being performed using the same protocol and under the same conditions as described at length above in this example with equivalent quantities (EU) of the Myrothecium verrucaria bilirubin oxidase), showed that no precipitation arc was observed, which has been identified as being typical of an absence of identity (see Clausen, supra, at pages 144-146, § 6.1.2.1). Thus, the M. verrucaria bilirubin oxidase and the S. parvispora phenol oxidizing enzyme are neither wholly (nor partially) immunochemically identical.

The results of Slide D (which involve the same test being performed using the same protocol and under the same conditions as described at length above in this example but with the phenol oxidizing enzyme or bilirubin oxidase and with the quantities (EU) of the phenol oxidizing enzyme and bilirubin oxidase noted above) showed that a precipitation arc was observed in the well (well 4) which contained the S. parvispora phenol oxidizing enzyme and the M. verrucaria bilirubin oxidase (in addition to well 1 and 2 but not 3), thereby confirming that the observation of a lack of a precipitation arc between in slide 3 was not the result of inhibition owing to something other than phenol oxidizing enzyme. Thus, this slide and the results

thereof, confirm that the M. verrucaria bilirubin oxidase and the S. parvispora phenol oxidizing enzyme are neither wholly (nor partially) immunochemically identical.

EXAMPLE 11

Dye Transfer Prevention.

The potential of the enzymatic system to prevent dye transfer was assessed by washing a colored swatch in the presence of a white pick-up swatch. The experiments were performed in 25 ml carbonate buffer, pH 9, containing the two swatches of 5x5cm. The enzyme was dosed as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) units. One ABTS unit is defined as the amount of enzyme which an optical density increase of 1 OD/min at 418 nm in the presence of 2 mM ABTS in 20 mM Tris buffer, pH 9. Experiments were performed in the presence of 0 units (u), 0.5 u, 1 u, and 2 u/ml of wash solution. Phenothiazine-10-propionate was added as an enhancer of theenzyme activity. This enhancer was added at concentrations of 0 µM, 50 µM, 100 µM and 250 µM. The fabrics were agitated in the wash solution for 30 minutes. Afterwards, they were tumble dried and the reflectance spectra were measured using a Minolta spectrometer. The data thereby obtained were transferred to the CIELAB L*a*b* color space parameters. In this color space, L* indicates lightness and a* and b* are the chromaticity coordinates.

The color differences between the control swatch, without addition of the enzymatic bleach system, and the swatch washed in the presence of the enzyme and/or phenothiazine-10-propionate, was expressed as ΔE, calculated from the following equation:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

The whiteness (ΔL) and the color difference (ΔE) obtained by the above method are given in the table below.

	Reactive Black 5		Direct Green 26	
	ΔL	ΔE	ΔL	ΔE
Enzyme: 0 unit PTP: 0 µM	0	0	0	0
Enzyme: 0.5 unit PTP: 0 µM	1.6	1.7	-0.4	0.7

	Reactive Black 5		Direct Green 26	
	ΔL	ΔE	ΔL	ΔE
Enzyme: 1 unit PTP: 0 μM	2.6	2.7	-0.1	0.5
Enzyme: 2 unit PTP: 0 μM	3.0	3.1	0.1	0.3
Enzyme: 0 unit PTP: 50 μM	-0.4	0.4	0	0.3
Enzyme: 0.5 unit PTP: 50 μM	4.1	4.3	1.9	2.6
Enzyme: 1 unit PTP: 50 μM	5.1	5.2	1.9	3.0
Enzyme: 2 unit PTP: 50 μM	5.2	5.3	3.0	3.9
Enzyme: 0 unit PTP: 100 μM	-1.4	1.5	0.1	0.4
Enzyme: 0.5 unit PTP: 100 μM	4.3	4.5	2.2	3.1
Enzyme: 1 unit PTP: 100 μM	5.2	5.2	2.5	3.5
Enzyme: 2 unit PTP: 100 μM	4.8	4.9	2.7	3.7
Enzyme: 0 unit PTP: 250 μM	-1.2	1.3	0.5	0.5
Enzyme: 0.5 unit PTP: 250 μM	5.1	5.2	2.1	3.1
Enzyme: 1 unit PTP: 250 μM	5.5	5.6	2.3	3.7
Enzyme: 2 unit PTP: 250 μM	5.3	5.4	2.4	3.9

EXAMPLE 12

Bleaching of Tomato Stains.

The ability of a phenol oxidizing enzyme of the present invention to bleach stains was assessed by washing cotton swatches soiled with tomato paste in the presence of Stacchybotrys chartarum phenol oxidizing enzyme (which is obtainable by the methods disclosed in Example 4 and 5) and an enhancer. The experiments were performed in 15 ml borate buffer, pH 9, and phosphate buffer, pH 7. The enzyme was dosed as ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) units. One ABTS unit is defined as the amount of enzyme which an optical density

increase of 1 OD/min at 418 nm in the presence of 2 mM ABTS, in 20 mM Tris buffer, pH 9. Experiments were performed in the presence of 2.8 units/ml of wash solution.

Phenothiazine-10-propionate was added as an enhancer of the enzyme activity. This enhancer was added at concentrations of 250 μM. The swatches were washed during 30 minutes, at 30 °C. After the wash, the residual color of the stains was measured as in Example 11. In the table below the difference in color measurement is given between the stain before and after the wash.

Wash condition	ΔE
no enzyme, 250 μM PTP, pH 7	11.5
2.8u enzyme, 250 μM PTP, pH 7	16.7
no enzyme, 250 μM PTP, pH 9	11.4
2.8u enzyme, 250 μM PTP, pH 9	15.2

As can be seen from the ΔE values, the bleaching of the tomato stain is improved in the presence of the enzyme preparation.

Example 13

Amino Acid Sequence Analysis of Stachybotrys chartarum Phenol Oxidizing Enzyme

Stachybotrys chartarum phenol oxidizing enzyme prepared as disclosed in Example 4 was subjected to SDS polyacrylamide gel electrophoresis and isolated. The isolated fraction was treated with urea and iodoacetamide and digested by the enzyme endoLysC. The fragments resulting from the endoLysC digestion were separated via HPLC (reverse phase monobore C18 column, CH3CN gradient) and collected in a multititer plate. The fractions were analysed by MALDI for mass determination and sequenced via Edman degradation. The following amino acid sequences were determined and are shown in amino terminus to carboxy terminus orientation:

N' DYYFPNYQSARLLXYHDHA C' (SEQ ID No:7)

N' RGQVMPYESAGLK C' (SEQ ID No:8)

Figures 4A-4B is an amino acid alignment of the Stachybotrys chartarum phenol oxidizing enzyme fragments with Myrothecium verrucaria bilirubin oxidase and LEPTOTHRIX DISCOPHORA manganese oxidizing protein.

Example 14

Cloning Genomic Nucleic Acid

Two degenerated primers were designed based on the peptide sequence. (SEQ ID NO:9) Primer 1 contains the following sequence: TATTACTTTCCNAAYTAYCA where N represents a mixture of all four nucleotides (A, T, C and G) and Y represents a mixture of T and C only. Primer 2 contains the following sequence: TCGTATGGCATNACCTGNCC. (SEQ ID NO:10)

For isolation of genomic DNA encoding phenol oxidizing enzyme, DNA isolated from Stachybotrys chartarum (MUCL # 38898) was used as a template for PCR. The DNA was diluted 100 fold with Tris-EDTA buffer to a final concentration of 88 ng/ul. Ten microliter of diluted DNA was added to the reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer, 0.296 microgram of primer 1 and 0.311 microgram of primer 2 in a total of 100 microliter reaction. After heating the mixture at 100°C for 5 minutes, 2.5 units of Taq DNA polymerase was added to the reaction mix. The PCR reaction was performed at 95°C for 1 minute, the primers were annealed to the template at 45°C for 1 minute and extension was done at 68°C for 1 minute. This cycle was repeated 30 times to achieve a gel-visible PCR fragment. The PCR fragment detected by agarose gel contained a fragment of about 1 kilobase which was then cloned into the plasmid vector pCR-II (Invitrogen). The 1 kb insert was then subjected to nucleic acid sequencing. The sequence data revealed that it was the gene encoding Stachybotrys chartarum because the deduced peptide sequence matched the peptide sequences disclosed above sequenced via Edman degradation. The PCR fragments containing the 5' gene and 3' gene were then isolated and sequenced. Figure 6 provides the full length genomic sequence (SEQ ID NO:3) of Stachybotrys oxidase including the promoter and terminator sequences.

Example 15

Cloning the cDNA encoding Stachybotrys phenol oxidizing enzyme

Stachybotrys chartarum strain (MUCL 38898) was grown in laccase production medium and RNA was extracted from mycellium and used as a template for cDNA isolation. The total cDNA was synthesized by reverse transcriptase using 4.3 microgram of RNA in a 20 microliter reaction containing 0.34 microgram oligo dT_{18} primer, 0.5 mM of each of four nucleotides (A, G, C and T), 20 units of RNA inhibitor and 100 units of reverse transcriptase. The cDNA encoding the Stachybotrys phenol oxidizing enzyme was then cloned by PCR in two steps. First, the 5 ' cDNA was cloned as a 678 bp fragment using the following two primers: GTCAATATGCTGTTCAAG (SEQ ID NO:11) and CTCGCCATAGCCACTAGG (SEQ ID NO:12). Second, the 3' cDNA was cloned as a 1301 bp fragment using following two primers: CTTTCGATGGTTGGGCTG (SEQ ID NO:13) and GTTCTAGACTACTCCTCGATTCCAAGATC. The cDNA sequence of 1791 bp is shown in Figure 5.

Example 16

Comparison of the Stachybotrys chartarum phenol oxidizing enzyme genomic DNA and cDNA

A comparison of the cDNA with genomic DNA revealed that there were five introns in the genomic DNA. The protein translation start site (ATG) is at nucleotide #1044 to #1046 and the translation stop site is at nucleotide #3093 to #3095. Protein sequence translated from cDNA and genomic DNA contains 594 amino acids.

Comparison of the Stachybotrys chartarum phenol oxidizing enzyme with other oxidizing enzymes

The protein sequence SEQ ID NO:2 was used as query to search GCG (Genetics Computer Group University Research Park, Madison Wisconsin) DNA and protein databases. It showed that Stachybotrys oxidase shared 60 % identity to bilirubin oxidase at the protein sequence level. Figure 7 shows the sequence alignment of the two proteins.

Example 17

Expression of Stachybotrys phenol oxidizing enzyme in Aspergillus niger var. awamori

The DNA fragment containing nucleic acid encoding the Stachybotrys phenol oxidizing enzyme flanked by two newly introduced restriction enzyme sites (Bgl II and Xba I) was isolated by PCR (Figure 9). This PCR fragment was first cloned into

the plasmid vector pCR-II and subjected to nucleic acid sequencing to verify the gene sequence. This DNA fragment was then cloned into the Bgl II to Xba I site of vector (pGAPT, see Fig 8). The vector used for expressing the Stachybotrys phenol oxidizing enzyme contains the Aspergillus niger glucoamylase gene promoter (from bases 1 to 1134) and terminator (from bases 1227 to 1485), a multicloning site (from bases 1135 to 1227), Aspergillus nidulans pyrG gene (from bases 1486 to 3078) as selection marker for fungal transformation and puc18 plasmid backbone for propagation in E. coli. The expression plasmid designated as pGAPT-gDO104 was then transformed into Aspergillus (strain dgr246:p2, Appl. Micro. Biotechnol, 1993, 39:738-743) by standard PEG methods. Transformants were selected on plates without uridine. Forty transformants were grown on CSA plates and then transferred to shake flasks containing CSL special medium with maltose. CSA plates contain: NaH2PHO4*H2O: 1 g/l; MgSO4: 1g/l; Maltose: 50g/l; Glucose: 2g/l; Promosoy: 10g/l; Mazu: 1 ml/l; and Bacto Agar: 15g/l. CSL medium is described in Dunn-Coleman et al., 1991, Bio/Technology 9:976-981. CSL special medium is CSL medium with the glucose and fructose eliminated. ABTS assays were performed at days 3, 6, and 10. The transformants were also grown in CSL first and then transferred after 1 day's growth to Clofine-special medium. After 6 days growth, these samples were assayed for ABTS activities (>0.2 units). Five best transformants were spore purified and tested again for ABTS activity (>5 units/ml) after 8 day growth in Clofine medium. Figure 10 shows a SDS-protein polyacrylamide gel indicated the expression level of the recombinant Stachybotrys oxidase in Aspergillus niger var. awamori grown of a 6 day culture grown in CSL special medium.

Example 18

Expression of Phenol oxidizing enzyme in Trichoderma reesei:

The expression plasmid for use in transforming Trichoderma reesei was constructed as follows. The ends of the BglII to XbaI fragment shown in Figure 9 containing the gene encoding the Stachybotrys phenol oxidizing enzyme were blunted by T4 DNA polymerase and inserted into PmeI restriction site of the Trichoderma expression vector, pTrex, which is a modified version of pTEX, see PCT Publication No. WO 96/23928 for a complete description of the preparation of the pTEX vector, which discussion is herein incorporated by reference, which

contains a CBHI promoter and terminator for gene expression and a Trichoderma pyr4 gene as a selection marker for transformants. The linear DNA fragment containing only the CBH1 promoter, the Stachybotrys phenol oxidizing gene, the CBH1 terminator and selection marker pyr4 was isolated from a gel and was used to transform a uridine auxotroph strain of Trichoderma reesei (see United States Patent no. 5,472,864) which has the four major cellulase genes deleted. Stable transformants were isolated on Trichoderma minimal plates without uridine. The transformants were grown on 50 ml of Proflo medium in shake flasks for 7 days at 28°C to 30°C and expression of the phenol oxidizing enzyme was assayed by ABTS (> 0.2 units/ml) and SDS-PAGE protein gel. Proflo medium is composed of (g/l) Proflo 22.5; lactose 30.0; $(NH_4)_2SO_4$ 6.5 KH_2PO_4 2.0; $MgSO_4.7\ H_2O$ 0.3; $CaCL_2$ 0.2; $CaC0_3$ 0.72; trace metal stock solution 1.0 ml/l and 10% Tween 80 2.0 ml/l. The trace metal stock solution used had (g/l) $FeSO_4.7H_2O$ 5.0; $MnSO_4.H_2O$ 1.6; $ZnSO_4.7H_2O$ 1.4; $CoCl_2.6H_2O$) 2.8.

Example 19

Expression of Stachybotrys phenol oxidizing enzyme in Saccharomyces cerevisiae:

The BglII to XbaI fragment of the cDNA (SEQ ID NO:1) of the phenol oxidizing gene was cloned into yeast expression vector yES2.0 (Invitrogen) which contains the yeast Gal 1 promoter and Cyc 1 terminator, to control expression of the phenol oxidizing gene, and the yeast URA3 gene as a selection marker. The expression plasmid was transformed into a yeast strain (Invitrogen Sc2 strain). The transformants were selected on yeast minimal plate without uridine. Four randomly picked transformants showed activity in plate assay (colored halo formation in yeast minimal plate with 1mM ABTS) while the control plasmid vector did not show any colored halo formation. All references and publications disclosed herein are hereby incorporated by reference in their entirety.